Amino Acid Compositions of Proteinoids

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by

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SUMMARY

Proteinoids prepared by thermal copolymerization of eighteen common amino acids have been analyzed. Conditions for quantitative recovery of the total amino acids following hydrolysis have been established. The effects of various amino acid reaction mixtures upon the composition of the polymers obtained have been studied.

Simultaneous polycondensation of the eighteen amino acids commonly found in protein is accomplished by heating at 150-200° dry mixtures containing a sufficient proportion of dicarboxylic amino acid (1, 2). Typically, four moles of aspartic acid per mole of each other amino acid will yield after dialysis workable amounts of such polymers. It is also possible to condense thermally all of the common amino acids by using sufficient lysine (3, 4). Polyphosphoric acid may be added to enhance yield (5) and to reduce temperatures required for condensation with dicarboxylic amino acid (6, 7).

The polymers thus obtained are found to have the same qualitative amino acid composition as proteins, to fall into the lower end of the range of molecular weight of proteins, and to be protein-like in many other properties (1, 2, 4, 8-10). The complement of amino acids and the molecular weight are the first standards (11, 12) in descriptive definitions of proteins, of which the proteinoids are models. The quantitative compositions are also of interest in these relationships.

Further tests of the inference of internal ordering in proteinoids (1, 2, 13), the compositions yielding various regular microparticles (7), and the effects of content of proteinoids of basic and acidic types on Gram-positive or Gram-negative (14) stains also need data on amino acid composition. When samples of these polymers have been hydrolyzed for analysis by procedures usual for protein, total recoveries of amino acids have often been less than 100% of that expected from pure polyamino acid. Accordingly a systematic study of effects of purification and time of hydrolysis upon total recovery was initiated. In the course of such studies, a finer understanding of the effect of temperature of hydrolysis has been achieved.

The experimental results, embodied in this paper, indicate that purity of polyamino acid, temperature, and length of time of hydrolysis can each contribute significantly to variations in total recovery of amino acids. In order to obtain the precise results needed for quantitative balance sheets, it is not sufficient simply to apply conditions of hydrolysis which are known to be adequate for the analytical hydrolysis of contemporary proteins (cf. 7).

MATERIALS AND METHODS

2:2:1-Proteinoid, 2:2:2-Proteinoid, 2:2:3-Proteinoid, and 2:2:4-Proteinoid- The procedure described earlier (2) for Mixture A was followed. The 2:2:1 designation signifies proportions by weight of 2 DL-aspartic acid, 2 L-glutamic acid, and 1 of an equimolar mixture of the other sixteen amino acids commonly found in protein. For other proteinoids, the figures correspond to the same components. The hot solution of amino acids in pyroglutamic acid was maintained at 170° for 6 hours except for the preparation used for Table III, which was heated 10 hr. A convenient method of further purification was simple fractionation from hot water by the method used in producing microspheres from proteinoid (15). An example is given.

Water (140 ml.) was boiled with 6.0 g. of crude 2:2:3-proteinoid (6.0 g.) for 2 min. The hot liquid was filtered from undissolved material, and the resultant filtrate in which spherules separated was dialyzed for 4 hr. and then lyophilized. The purified fraction was 1.09 g. More of soluble proteinoid could be obtained by further leaching with hot water of the undissolved polymer.

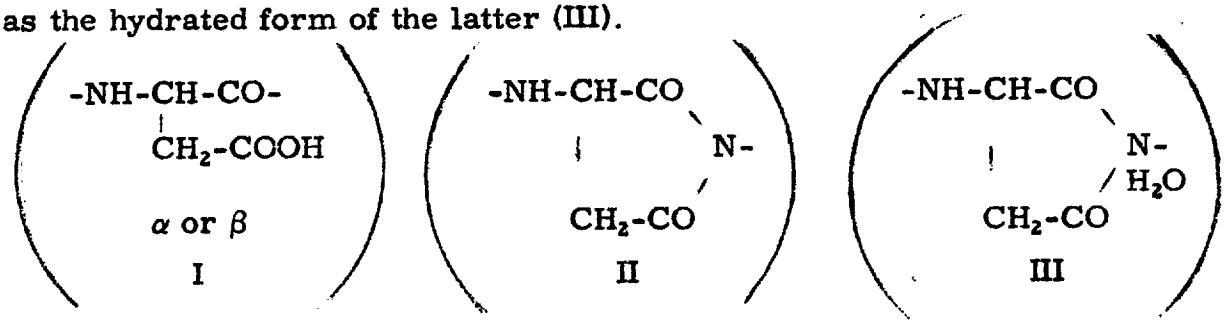
Some of the purified material (0.50 g.) was treated in the same way with 12 ml. of hot water with a little charcoal added before filtration. Dialysis was for 1 hr. The lyophilizate weighed 0.30 g.

Analysis - Each sample was dried at 100° in an Abderhalden pistol for 5 hr. The typical preparation for analysis consisted of placing 15 mg. of dried polymer in 6.0 ml. of 6 N redistilled HCl in a sealed tube, which had been flushed with nitrogen gas, and heating at 105° ± 2°, or at 110° ± 2°. The product of this hydrolytic treatment was concentrated to dryness in a vacuum desiccator over sodium hydroxide. The residue was dissolved in a measured amount of water and the solution was adjusted to pH 2.2. In those cases in which hydrolysis was performed in one laboratory and the analysis in another, the solution was diluted to 10 ml. in an ampul which was then sealed and heated in boiling water for 30 minutes in order to sterilize it.

Measured volumes equivalent to approximately 2 mg. of proteinoid were applied to chromatographic columns for automatic analysis. For some samples in which there was a preponderance of aspartic acid, an additional analysis of 1/10 or 1/20 the volume for the initial run was made on a column 0.9 x 5 cm equilibrated with 0.2 M citrate buffer, pH 3.24. Identification of amino acids in the resulting recordings was based principally on their chromatographic behavior in the system used for analysis as compared with well established patterns for standard mixtures. In some instances the relative colorimetric absorbancies of the ninhydrin reaction products at 570 mμ and at 400 mμ provided additional information useful for identification.

Assays were performed by the chromatographic methods of Spackman, Stein, Moore (16) with the use in some cases of a constructed automatic amino acid analyzer (17) and in other cases of a Phoenix Model K-5000 Analyzer.

The aspartic acid content can be calculated on the basis of aspartyl residues in peptide linkage (I), aspartyl residues in imide linkage (II), or as the hydrated form of the latter (III).



The calculations based on I and III are equal. Since "polyaspartic acid" retains its hydration on drying under the conditions employed for proteinoid in this study (18), Type III was assumed for the calculations.

In Tables I, II, and III values for serine and threonine are not recorded inasmuch as these are trace amounts. The value for tryptophan is also missing from these tables, tryptophan having been destroyed by mineral acid hydrolysis.

RESULTS AND DISCUSSION

The recording depicted in Figure 1 reaffirms by a quantitative tracing the qualitative observation that all of the amino acids common to protein can under appropriate conditions of heating be simultaneously condensed into a single polymeric preparation (1).

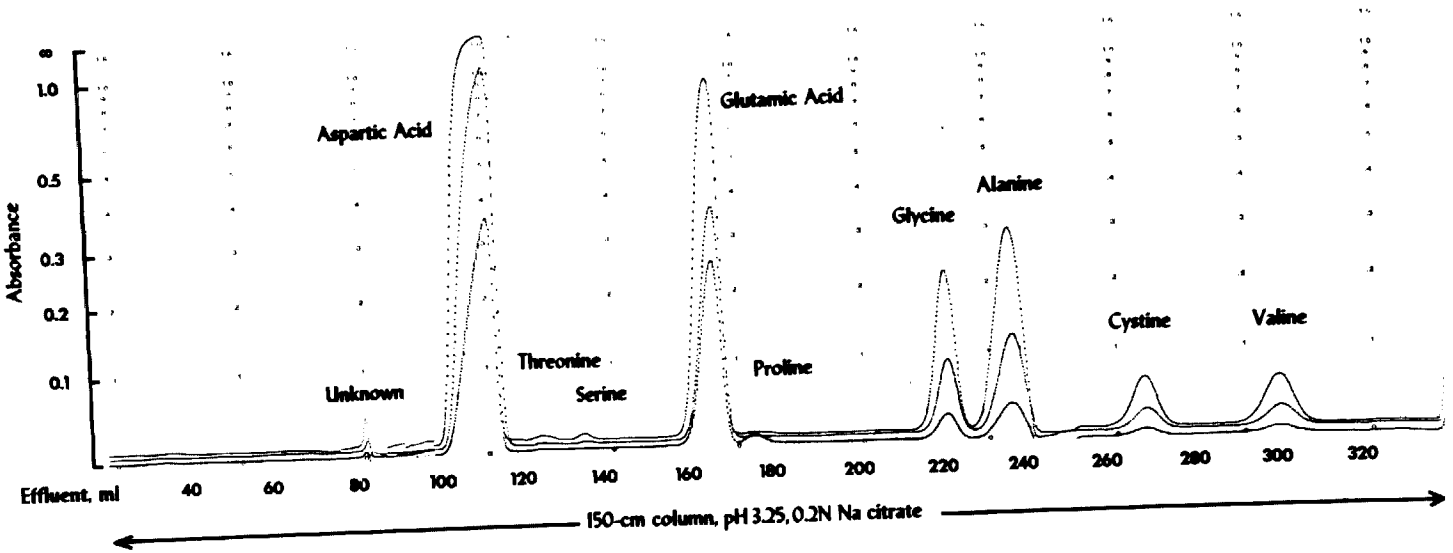
The proportions of serine and threonine found in this and other acid proteinoids made in essentially the same way are below those typical of proteins. The qualitative inclusion of these amino acids has however been observed in at least five laboratories (1, 19, 20). Proportions of serine and threonine as high as 1.5% can be incorporated into simple thermal copolyamino acids under special conditions employing hypophosphorous acid.

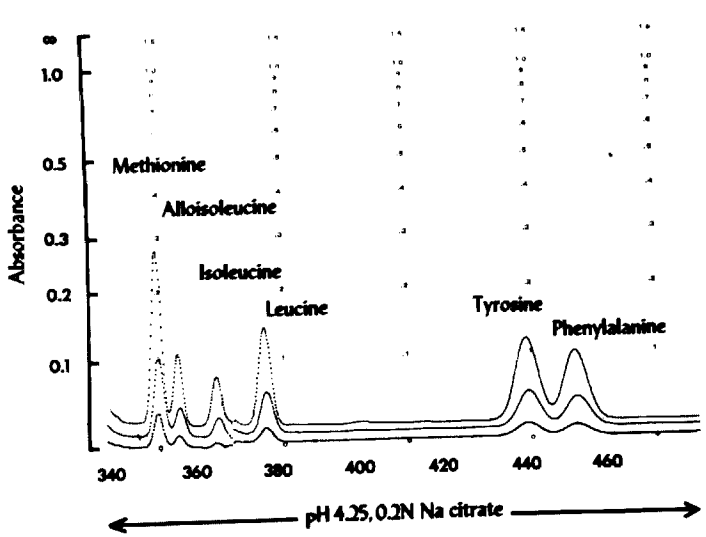
Although tryptophan is entirely missing from this chromatogram of a hydrochloric acid hydrolyzate, tryptophan has been shown to be present in intact proteinoids by color test and by microbial assay of the alkaline hydrolyzate (1, 2, 8, 19).

The recording of Fig. 1 is also quite free of unidentified peaks. The only unknown found in significant proportion in this analysis is that which appears at 82 ml. from the long column. This peak usually appears between 82 and 90 ml. Standards showed that the compound at this point is not taurine.

Other amino acids appear in proportions equal to or exceeding (aspartic acid) those reported for protein.

The peak labelled cystine may, however, be partly or mainly another material eluting at the same position as cystine. This possibility is apparent from the absorption ratio at 440/570 mμ and also from the expectation of a less symmetrical peak due to mesocystine which arises from cystine during acid hydrolysis. The relationships are being studied in copolymers of simpler composition (23) in which it is possible to convert the cystine quantitatively to cysteic acid. The appearance of alloisoleucine in the proteinoid product is due to the fact that the original isoleucine is an L-isoleucine/D-alloisoleucine pair.


Absorbance
∞
1.0
0.5
0.3
0.2
0.1
Aspartic Acid
Unknown
Threonine
Serine
Glutamic Acid
Proline
Glycine
Alanine
Cystine
Valine
Effluent, ml
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
150-cm column, pH 3.25, 0.2N Na citrate


Absorbance
∞
1.0
0.5
0.3
0.2
0.1
Methionine
Alloisoleucine
Isoleucine
Leucine
Tyrosine
Phenylalanine
340
360
380
400
420
440
460
pH 4.25, 0.2N Na citrate

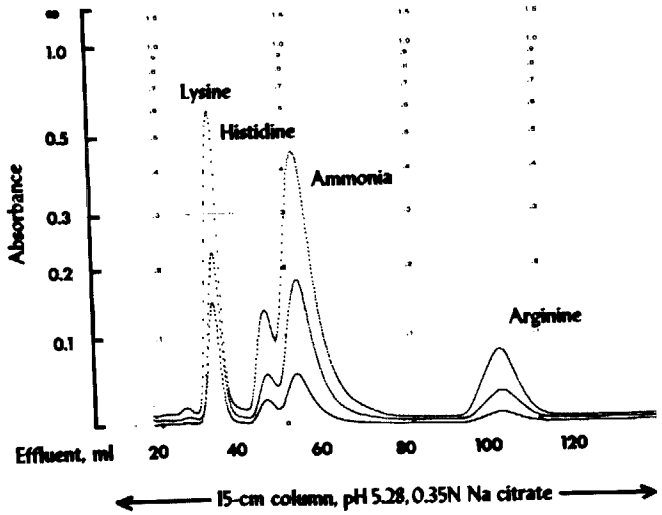

Absorbance
∞
1.0
0.5
0.3
0.2
0.1
Lysine
Histidine
Ammonia
Arginine
Effluent, ml
20
40
60
80
100
120
15-cm column, pH 5.28, 0.35N Na citrate

Figure 1. Chromatogram of hydrolyzate of 2:2:3-proteinoid as obtained on automatic amino acid analysis.

Substantial proportions of ammonia appear on the chromatogram despite the fact that neither ammonia as such nor amino acid amides were present in the reaction mixture. A possible source is the deamination of amino acids on heating (e.g. aspartic acid ——→ fumaric acid + ammonia) followed immediately by reaction of the ammonia with the imide linkages (4) present in the polymer before the polyimide is converted to polypeptide. The proteinoids may thus include amide groups such as found in protein even though asparagine and glutamine were not part of this mixture. They thus contain eighteen to twenty amino acids. They may also hold some ammonia as ammonium salt. Asparagine (24) glutamine (1), and, incidentally, pyroglutamic acid (25) can substitute for the corresponding dicarboxylic amino acid in reaction mixtures yielding thermal polyamino acids.

The chromatogram reveals aspartic acid and glutamic acid as the two principal components of the proteinoid and this fact is consistent with the inclusion of each to the extent of 29% by weight of the reaction mixture. The fact that aspartic acid is present in larger proportion is consistent with scores of analyses of simple and highly heterocompositional thermal polyamino acids. In each of these, aspartic acid is found to enter into combination at several times the proportion true for glutamic acid under analogous conditions. The other amino acids are present in the reaction mixture at a ratio of 2.9% each. The proportion of each found in the product is thus an index of the ease with which it copolymerizes. Lysine and alanine stand out in this property and the results in the 2:2:3-proteinoid typify their relative ease of incorporation under other sets of conditions. Lysine is, in fact, found to copolymerize yet more readily as the free base; the hydrochloride was used in this polymerization. Glycine is an amino acid capable of thermal homopolymerization (26); accordingly the fact that it is not incorporated in larger ratio is somewhat surprising.

In cystine assays, chromatograms of the hydrolyzate following performic

acid oxidation of the polymer have been obtained. These are very similar to that of Fig. 1 except that the cystine peak is smaller and a cysteic acid peak is present. The methionine peak is entirely replaced by one for methionine sulfone, and the relative size of the tyrosine peak has decreased to an absorbance approximately one-third below the 0.1 mark.

No regular or analytically significant increase or decrease in individual amino acid content is apparent in the data of Table 1. For quantitative total recovery a hydrolytic period longer than one day is needed. Otherwise, no indication of increased release during hydrolysis or of progressive destruction is found for the purified 2:2:1-proteinoid.

Comparison of these results with earlier analyses carried out on 24 hour hydrolyzates by a dinitrophenylation method can be drawn for contents of aspartic acid, glutamic acid, and the basic-neutral fraction of amino acids (2). The values by that technique for the unfractionated proteinoid similarly prepared at 170° were 65, 12, and 23% whereas for the purified and unpurified fractions as analyzed automatically the corresponding values are after three days of hydrolysis 66, 16, and 16% and 67, 12, and 21% respectively. The latter set of figures is more pertinent than those for the purified fraction since the largest fraction on purification is the residual part (MATERIALS AND METHODS). This relatively high concordance in two types of analysis bears special significance inasmuch as an inference of internal order in amino acid residues in the proteinoids rests upon the first type (2). This inference of internal order is essentially unchanged by the newer analysis.

In Table II are recorded the results of hydrolyzing a series of proteinoids prepared with varying proportions of basic-neutral amino acids, and analyzing for all amino acids except tryptophan, proline, serine, and threonine. The low proportions of each of these four were neglected in the computations of Tables I and II. All of these samples were hydrolyzed for 72 hours at 105°. The row of figures presenting recovery values indicates that total recovery of amino acids drops as the proportion of basic-neutral amino acids is

Table I

Analyses of Purified 2:2:1-Proteinoid after the Indicated Number of Days of Hydrolysis at 105°

Amino Acid	1	2	3	4	6
Lysine	1.58% [a]	1.64%	1.98%	1.79%	1.85%
Histidine	1.17	0.95	0.95	0.77	0.89
Ammonia	4.05	3.60	3.90	5.75	3.98
Arginine	1.06	0.94	0.93	0.90	0.98
Aspartic acid	59.8	66.0	65.6	63.6	64.3
Glutamic acid	15.5	15.8	15.9	15.2	15.8
Proline	[b]	0.28	0.36	0.28	0.31
Glycine	1.44	1.32	1.38	1.36	1.33
Alanine	2.60	2.30	2.56	2.43	2.51
Half-cystine	[c]	1.32	1.41	1.29	1.42
Valine	0.98	0.85	0.94	0.89	0.98
Methionine	1.05	0.94	0.99	1.04	1.08
Isoleucine	0.97 [d]	0.86	1.00	1.27	1.05
Leucine	0.95	0.88	0.96	0.93	0.96
Tyrosine	1.15	0.94	1.02	1.02	1.06
Phenylalanine	2.05	1.84	1.92	1.80	1.93
Total recovery	85 [e]	103	101	103	97

[a] Values are given in g. residues of amino acid / total g. residues.

[b] In this chromatogram, the peak for proline was not distinct.

[c] Half-cystine values may be partly other material.

[d] Isoleucine includes alloisoleucine.

[e] Total recovery = total g. residues of amino acid / wt. of polymer.

Table II

Composition of Proteinoid Hydrolyzates (105°, 3 days)

Prepared from Different Proportions of Amino Acids

Amino Acid	2:2:1	2:2:2	2:2:3	2:2:4
Lysine	1.89% [a]	3.34%	5.59%	6.16%
Histidine	0.94	1.41	1.97	2.42
Ammonia	3.82	3.78	6.53	8.92
Arginine	0.79	0.72	1.87	2.42
Aspartic acid	69.2	62.0	50.4	42.1
Glutamic acid	13.9	12.6	11.5	12.6
Glycine	1.19	2.09	2.99	3.82
Alanine	2.27	3.58	4.31	4.32
Half-cystine [b]	1.19	1.69	2.33	2.72
Valine	0.78	1.14	1.81	1.71
Methionine	0.85	1.71	2.30	2.72
Isoleucine [c]	0.90	1.27	1.77	2.14
Leucine	0.80	1.16	1.64	1.98
Tyrosine	0.53	1.09	1.99	2.21
Phenylalanine	1.10	1.97	3.38	4.04
Total Recovery [d]	85	82	67	57

[a] Values are given in g. residues of amino acid / total g. residues.

[b] Half-cystine may be partly other material.

[c] Isoleucine includes alloisoleucine.

[d] Total recovery = total g. residues of amino acid / wt. of polymer.

increased in the reaction mixture. Yield of product also decreases (2). The table as a whole demonstrates the variability in individual amino acid content in proteinoid as its ratio to dicarboxylic amino acid is varied. Such control of content is consistent with the results of an intensive study of contents of histidine reported earlier (4). As the ratio of individual amino acid to aspartic acid plus glutamic acid is progressively doubled, tripled, and quadrupled, the proportion of the amino acid is increased in almost every case. The tyrosine and phenylalanine contents are approximately quadrupled, although analyses in Tables I and II suggest that these values in 2:2:1-proteinoid in this series are low. Lysine, histidine, arginine, glycine, half-cystine, and methionine values are approximately tripled, while those for ammonia, alanine, valine, isoleucine, and leucine are approximately doubled. Aspartic acid decreases significantly, whereas glutamic acid content holds close to constant.

The results in Table III indicate that quantitative recovery of amino acids from a thermal proteinoid is possible even though the material examined is almost half composed of neutral and basic amino acids. The largest changes appear to be an increase in alanine content and a decrease in half-cystine content. The quantitative total recovery value fails to reflect the tryptophan content. The proportion of tryptophan is typically 1.0% in a 2:2:1-proteinoid (2); such a figure would not appreciably alter the total recovery values in Table III. The fact that recovery (Table III) is 100% from the repurified polymer rules out the possibility of significant proportions of bound compounds other than amino acids, such as fumaric acid. Non-amino acid constituents, e.g., guanine (27), are present prior to purification.

The composition of the proteinoid following each of two successive purifications from hot water is very similar to that prior to purification, despite much opportunity for fractionation during the purification. The polymeric mixture is, accordingly, not a mixture of homopolymers since such a mixture should be fractionated under these conditions.

The negligible degree of change in composition upon successive purifications,

Table III

Composition of Hydrolyzates (110°, 4 days) of 2:2:3-Proteinoid Following One and Two Purifications

Amino Acid	Unpurified	Purified	Repurified
Lysine	5.1% [a]	5.4%	5.4%
Histidine	1.8	2.0	2.0
Ammonia	8.6	8.1	6.9
Arginine	2.0	2.3	2.4
Aspartic acid	51.7	50.2	51.1
Glutamic acid	10.7	11.6	12.0
Proline	0.7	0.6	0.6
Glycine	2.7	3.1	2.8
Alanine	4.0	4.3	5.5
Half-cystine [b]	4.5	3.5	3.4
Valine	1.2	1.2	1.2
Methionine	1.8	1.9	1.7
Isoleucine [c]	1.2	1.3	0.9
Leucine	1.3	1.2	1.1
Tyrosine	2.0	1.9	1.7
Phenylalanine	1.8	1.7	1.5
Total Recovery [d]	84.8%	97.5%	100.0%

[a] Values are given in g. residues of amino acid / total g. residues.

[b] Half-cystine values may be partly other material.

[c] Isoleucine includes alloisoleucine.

[d] Total recovery = total residues of amino acid / wt. of polymer.

together, with studies by electrophoresis (2), ultracentrifugal analysis (28), fractionation by ion exchange chromatography (12), and studies of positional distribution of amino acid residues (2, 13) indicate that the degree of heterogeneity of the proteinoid molecule is limited. The concepts of randomness and order are abstruse (29, 30); however, a statistically random polymer is ruled out by the data. The possibility of internally governed order in the proteinoids can be understood on the basis that each amino acid has its steric and electronic individuality which must be expressed in individual rates of reaction at each stage of the growing peptide chains.

The linkages in the proteinoids may involve the β and γ carboxyl groups of the dicarboxylic amino acids, ϵ-amino groups of lysine, and various additional interactions other than peptide linkages. Proteinoids are however susceptible to attack by proteolytic enzymes (2, 8, 19) and they can be used as partial peptone substituents in bacterial media (2). The repeatability and controllability of the compositions have also been reported from another laboratory (9).

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